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During anaphase of mitosis, sister chromatids are separated by the mitotic spindle. The spindle assembly checkpoint protects the integrity of the genome by initiating a cell cycle delay if chromosomes are not properly attached to the spindle. Cells lacking a functional spindle checkpoint may gain or lose genetic information, which can cause cell death or predispose cells to cancer. For example, loss of checkpoint function has been observed in human cancer cell lines, and decreased expression of the checkpoint component, *hsMAD2*, has been demonstrated in human breast cancers. Most human spindle checkpoint components were identified by their similarity to yeast checkpoint proteins that were discovered through genetic screens. Many aspects of spindle checkpoint function are not yet understood, and genetic evidence indicates there are additional checkpoint proteins that have not been identified. This project aims to use genetic screens in fission yeast to identify and characterize novel components of the yeast and animal spindle checkpoint pathways and novel mutant alleles of known yeast spindle checkpoint genes. To date, mutations in three known yeast spindle checkpoint genes and a mutation in a potentially novel component of the yeast checkpoint pathway have been identified. A second genetic screen has been initiated to identify mouse cDNAs which induce a metaphase arrest in fission yeast and may encode spindle checkpoint proteins. The genes identified by these studies will be used to further elucidate the mechanism of spindle checkpoint function.

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INTRODUCTION

For cells to proliferate, they must completely duplicate their genetic material during S phase of the cell cycle and then partition one complete set of chromosomes into each of the daughter cells during anaphase. Failure to achieve equal segregation of the chromosomal DNA can lead to genetic loss, lethality, or aneuploidy. This aneuploidy is a feature of cancer cells which typically correlates with aggressive tumors and a poor prognosis (Hartwell and Kastan, 1994). Genetic loss and aneuploidy occur rarely because eukaryotic cells have checkpoints to monitor such critical events in the cell cycle and ensure that downstream events can not proceed until the successful completion of upstream events (Hartwell and Weinert, 1989). The spindle assembly checkpoint system ensures that chromosome segregation is not initiated before the chromosomes are attached to a properly assembled bipolar mitotic spindle (Wells and Murray, 1996). When defects are detected, the checkpoint system blocks the cell cycle at metaphase, the stage of mitosis in which the sister chromatids align at the equatorial plate. This delay allows time for spindle repair, thus preventing the gain or loss of chromosomes in the daughter cells. Cells mutated in spindle checkpoint genes fail to arrest the cell cycle in response to spindle damage because they fail to detect the defect or because they can not restrain the cell cycle. These cells undergo an aberrant division that results in missegregation of DNA and loss of genetic information (Millband et al., 2002). Loss of the spindle checkpoint may result in the multiple genetic changes that mark the development of tumor cells (Cahill et al., 1998; Lengauer et al., 1998; Orr-Weaver and Weinberg, 1998; Wassmann and Benezra, 2001). In support of this prediction, loss of checkpoint function has been observed in many human cancer cell lines (Cahill et al., 1998; Iwanaga et al., 2002; Ru et al., 2002; Takahashi et al., 1999), and decreased expression levels of the checkpoint component, *hsMAD2*, have been demonstrated in human breast cancers (Li and Benezra, 1996) and shown to promote lung tumors in mice (Michel et al., 2001).

The first spindle checkpoint genes were identified by two independent screens in the budding yeast, *Saccharomyces cerevisiae* (Hoyt et al., 1991; Li and Murray, 1991) These MAD and BUB screens identified six spindle checkpoint mutants by their sensitivity to microtubule destabilizing drugs. The *mad* and *bub* mutants are sensitive to microtubule disruption because they fail to arrest the cell cycle in response to this damage, resulting in missegregation of chromosomes and loss of genetic material. The only known human genes that encode components of the spindle checkpoint signaling pathway, such as *hsMAD2*, were identified based on sequence similarity to the yeast proteins (Cahill et al., 1999; Fisk and Winey, 2001; Jin et al., 1998; Li and Benezra, 1996; Ouyang et al., 1998; Stucke et al., 2002; Taylor et al., 1998). The fission yeast, *Schizosaccharomyces pombe*, is a useful system for discovering and characterizing components of the spindle checkpoint pathway because genetic approaches can be coupled with excellent cytology. Previous work in the Sazer laboratory identified the first two spindle checkpoint genes in *S. pombe*: *mad2* and *mph1* (He et al., 1998; He et al., 1997). Overexpression of either *mad2* or *mph1* activates the spindle checkpoint, arrests cells prior to anaphase, and is toxic to wild type cells. In the *S. pombe* spindle checkpoint pathway, *mph1* acts upstream of *mad2*. Therefore, while *mph1* overexpression prevents cell cycle progression in wild type cells, this toxic effect is not observed in *mad2* deletion mutants that lack a functional checkpoint. Based on this observation, a genetic screen was designed to identify new components of the spindle checkpoint pathway as suppressors of *mph1* overexpression toxicity. It is predicted that these new spindle checkpoint components will have mammalian homologues, and characterization of the *S. pombe* proteins will provide a better understanding of the spindle checkpoint pathway and may reveal new information about the process of tumorigenesis.

BODY

Specific Aim1: Identify and characterize additional components of the spindle checkpoint pathway in *S. pombe*.

The following tasks were outlined in the original Statement of Work for Specific Aim 1. Mutants have been isolated that will not arrest in response to *mph1* overexpression and are sensitive to mitotic spindle damage induced by the microtubule destabilizing drug, thiabendazole (TBZ) (Task 1). Therefore, these mutations will likely identify components of the spindle checkpoint pathway which act downstream of Mph1p. The genes mutated in selected strains will be identified (Task 4), and it will be confirmed that these genes function in the spindle checkpoint pathway (Task 5). These new checkpoint components will then be further characterized to determine where they act in the spindle checkpoint pathway (Task 6).

Summary of Work Described Previously Pertaining to Tasks 1-3 of Specific Aim 1:
To begin characterizing the strains from the *mph1* overexpression screen, I analyzed 17 highly TBZ-sensitive mutants that grow normally in the absence of TBZ. I subjected these strains to linkage analysis to determine if they are likely to be mutated in a known spindle checkpoint gene (Statement of Work, Task 3). I performed linkage analysis using null mutations of the known spindle checkpoint genes in *S. pombe*: *mad1*, *mad2*, *mad3*, *bub1*, and *bub3* (Bernard et al., 1998; He et al., 1998; He et al., 1997; Ikui et al., 2002; Millband and Hardwick, 2002).

Four of the strains from the *mph1* overexpression screen contain a mutation that is tightly linked to a known spindle checkpoint gene. Strain 1207 and strain 1221 contain mutations in a gene tightly linked to *bub1*. A plasmid carrying the *bub1* gene was able to confer TBZ resistance to strain 1207 and strain 1221, a confirmation that these strains are mutated in the *bub1* gene.

Strain 1208 contains a mutation tightly linked to the *bub3* gene. To confirm that strain 1208 is mutated in the *bub3* gene, I have shown that the 1208 mutation is unable to complement a *bub3* null mutation. Finally, strain 1201 contains a mutation that is tightly linked to *mad1*. These results indicate that the *mph1* overexpression screen has successfully identified three known spindle checkpoint genes, validating the strategy of the screen and making it likely that the screen will identify novel genes in this pathway.

Current Results Pertaining to Tasks 1-3 of Specific Aim 1:
Novel mutations in the known spindle checkpoint genes will provide new tools to further our understanding of how the spindle checkpoint is activated and how it initiates the cell cycle arrest (Statement of Work, Task 2). To date, the *mph1* overexpression screen has identified four strains that are mutated in known spindle checkpoint genes: 2 *bub1* mutants (bub1-1207 and bub1-1221), 1 *bub3* mutant (bub3-1208), and 1 *mad1* mutant (mad1-1201). I have amplified and sequenced the *bub1* alleles in bub1-1207 and bub1-1221 and the *bub3* allele in bub3-1208. The *bub1* gene in bub1-1207 contains a C to T nucleotide mutation that would result in an amino acid change at position 988, which introduces a premature stop codon in the highly conserved Bub1p protein kinase domain (see Figure 1). The *bub1* gene in bub1-1221 contains a C to T nucleotide mutation that would result in an amino acid change from alanine to valine at position 78, which maps inside the conserved Mad3-like region of Bub1p (see Figure 1). The *bub3* allele in bub3-1208 contains a G to A nucleotide mutation that would result in an amino

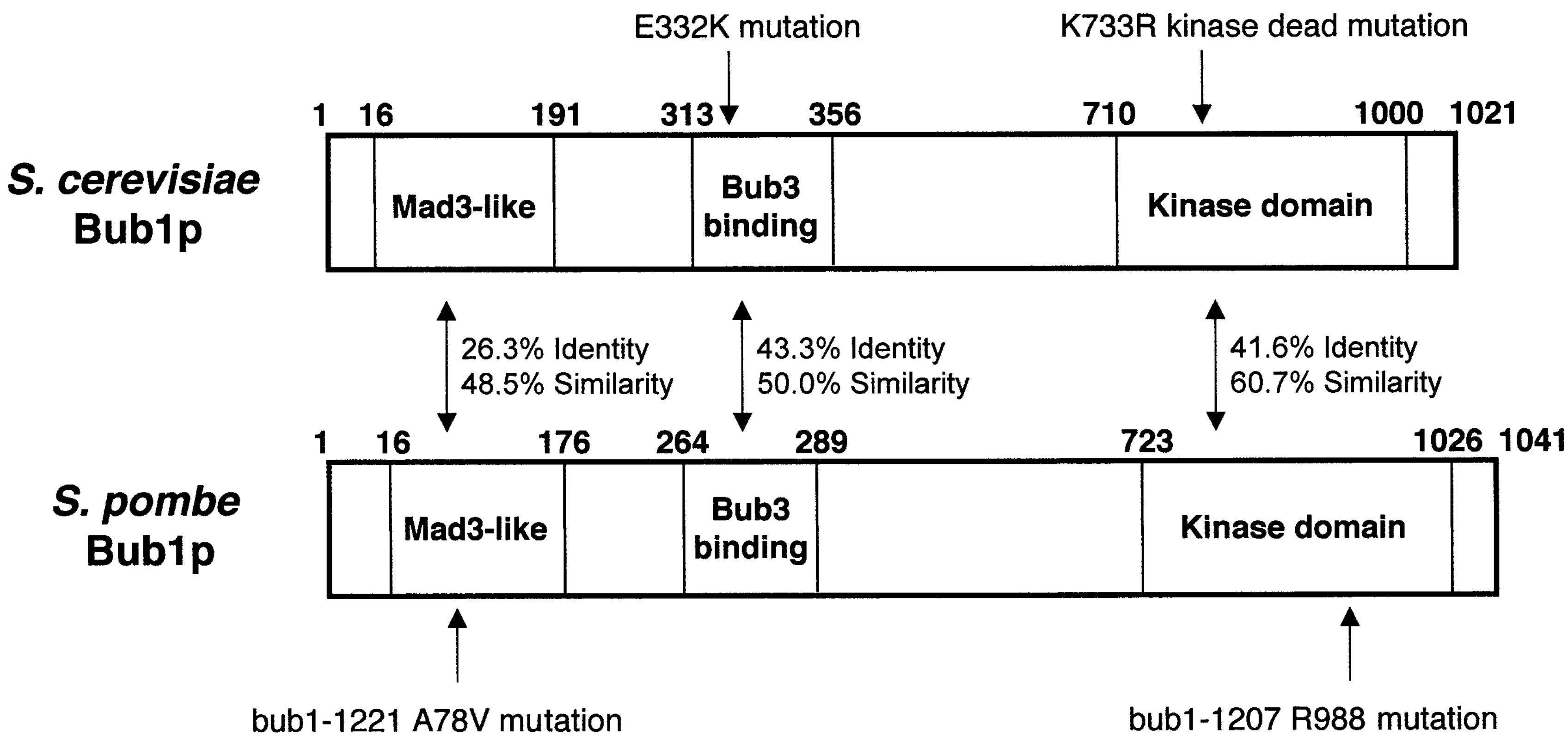


Figure 1: Two strains identified in the *mph1* overexpression screen are mutated in *bub1*. The *S.cerevisiae* and *S. pombe* Bub1 proteins are depicted illustrating known functional regions and the homology between *S. cerevisiae* Bub1p and *S. pombe* Bub1p in these functional regions. Arrows indicate locations of Bub1p mutations. The mutation in bub1-1221 occurs at amino acid 78 and maps inside the conserved Mad3-like region. The mutation in bub1-1207 occurs at amino acid 988 and maps inside the conserved kinase domain.

acid change from glycine to aspartic acid at position 255. The glycine at position 255 is conserved in *S. cerevisiae* Bub3p and human Bub3p and maps inside the most carboxy terminal WD repeat, which may be important for Bub3p interaction with other proteins (Taylor et al., 1998). I am currently amplifying and sequencing the *mad1* allele in mad1-1201.

The alleles of known spindle checkpoint genes identified in the *mph1* overexpression screen encode novel mutants of known spindle checkpoint genes and may provide useful tools to further characterize the spindle checkpoint pathway in *S. pombe*. Alternatively, the alleles may contain mis-sense mutations which do not produce a functional protein and therefore phenotypically resemble null mutations. To distinguish between these two possibilities, I compared the strains from the *mph1* overexpression screen which are mutated in known spindle checkpoint genes to their respective deletion mutants on plates containing TBZ. Only one mutant strain exhibited a phenotype that was distinct from its deletion mutant. The bub1-1207 strain is approximately 25 times more TBZ-sensitive than the bub1Δ strain (see Figure2). Since the bub1-1207 strain has a novel mutant phenotype, characterization of the checkpoint defect in this strain may further elucidate the role of Bub1p in the spindle checkpoint pathway.

The *bub1* gene in the bub1-1207 strain has a nucleotide mutation that would result in the premature introduction of a stop codon (see Figure 1). It is predicted that this mutant *bub1* allele would encode a Bub1 protein with 56 amino acids deleted from the carboxy terminus. This premature truncation occurs in the highly conserved protein kinase domain of Bub1p. This protein kinase domain is necessary for checkpoint activation in budding yeast (Roberts et al., 1994), but *in vivo* targets of the Bub1p kinase have yet to be identified.

The bub1-1207 strain may be useful in experiments addressing the importance of the Bub1p amino terminus in checkpoint function. Experiments performed in *Xenopus laevis* indicate that Bub1p^{Xl} is necessary for the recruitment of spindle checkpoint proteins to kinetochores that are not properly attached to the spindle, which occurs upon activation of the checkpoint (Sharp-Baker and Chen, 2001). However, Bub1p^{Xl} kinase activity is not required for the recruitment of checkpoint proteins to kinetochores because a Bub1p mutant which lacks kinase activity efficiently recruits checkpoint proteins to kinetochores. These results indicate that the Bub1p^{Xl} kinase domain mutant can still bind to other checkpoint effectors and proteins at the kinetochore. Perhaps the mutant Bub1p in the bub1-1207 strain interacts with other checkpoint proteins via its amino terminus but can not function in checkpoint activation because it lacks kinase activity due to the carboxy terminal truncation. Further experiments are required to better understand the checkpoint defect in bub1-1207. First, I will tag the *bub1* gene in bub1-1207 with HA at its endogenous locus at its carboxy terminus. The Hardwick lab has succeeded in tagging the wild type *S. pombe bub1* gene with HA at its carboxy terminus, and this tag did not interfere with the function of Bub1p (Bernard et al., 1998). To ensure that the HA tag does not alter the phenotype of bub1-1207, I will compare the TBZ-sensitivity of bub1-1207 to the HA tagged bub1-1207 strain. This tagged strain will then be used to confirm that Bub1p is expressed in the bub1-1207 mutant strain by Western blot analysis. The following strains expressing tagged versions of *S. pombe* spindle checkpoint proteins are available: GFP-mad1, GFP-mad2, GFP-mad3, GFP-bub1, and Myc-bub3 (Ikui et al., 2002; Millband and Hardwick, 2002; Hardwick, personal communication; Yanagida, personal communication). I will utilize these strains to perform co-immunoprecipitation experiments and determine if the mutant Bub1p has different binding partners than the wild type protein. I will also use these strains to determine if checkpoint

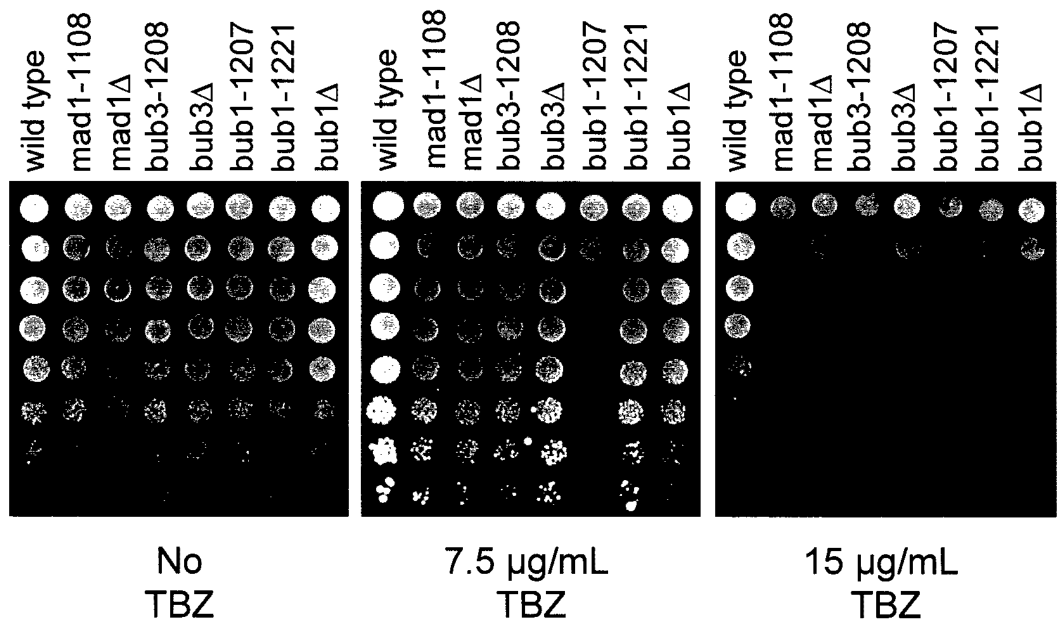


Figure 2: The bub1-1207 mutant from the *mph1* overexpression screen is more TBZ-sensitive than the *bub1Δ* strain. Serial dilutions of an equal number of cells were spotted on YE plates containing no TBZ, 7.5µg/mL TBZ, or 15µg/mL TBZ in a five fold dilution series starting with 10^6 cells. The TBZ-sensitivity of four strains from the *mph1* overexpression screen which are mutated in known spindle checkpoint genes were compared to their respective deletion mutants. The control plate with no TBZ was incubated for two days at 29°C to demonstrate equal spotting of strains. The plates containing TBZ were incubated for three days at 29°C.

proteins are recruited to kinetochores in the bub1-1207 mutant. No publications have reported the characterization of an *S. pombe bub1* mutant that, like bub1-1207, is phenotypically distinct from a bub1Δ strain. Since it is predicted that the Bub1p in bub1-1207 is truncated in the kinase domain, these studies will help determine if the *S. pombe* Bub1p kinase domain is necessary for Bub1p to interact with other checkpoint components and to bind to kinetochores. Based on studies in *Xenopus laevis*, it is predicted that the 1207 mutant Bub1p will be able to bind to Bub3p, and spindle checkpoint proteins will be efficiently recruited to kinetochores in this strain.

Since the bub1-1221 and bub3-1208 strains are not phenotypically distinct from a bub1Δ or a bub3Δ strain respectively, further characterization of the bub1-1221 and bub3-1208 strains may not contribute to our understanding of spindle checkpoint function. However, the identification of these strains from the *mph1* overexpression screen has revealed inactivating mutations of *S. pombe bub1* and *bub3*. The *bub1* gene in bub1-1221 is predicted to encode a protein that is mutated in the Mad3-like region of Bub1p. This region is conserved between *S. cerevisiae* Bub1p and *S. pombe* Bub1p, but the functional importance of this region is not yet understood. The *bub3* gene in bub3-1208 is predicted to encode a protein that is mutated in a highly conserved glycine residue which is located in the most carboxy terminal WD repeat domain. Bub3p has been isolated in spindle checkpoint protein complexes which include Mad1p, Mad2p, Mad3p, Cdc20p, and Bub1p (Brady and Hardwick, 2000; Fraschini et al., 2001; Roberts et al., 1994). It has been shown that the Bub3p WD repeat domains are required for its interaction with Mad2p, Mad3p, and Cdc20p, and mutation of one or more of the 3 WD repeat domains in *S. cerevisiae* Bub3p disrupts spindle checkpoint activation (Fraschini et al., 2001). Since bub1-1221 and bub3-1208 are as TBZ-sensitive as their respective deletion mutants, it is possible that the mutant proteins in these strains are not expressed in a stable form. As described for the bub1-1207 strain, I will determine if the mutant Bub1p in bub1-1221 and the mutant Bub3p in bub3-1208 are expressed by Western blot analysis. If these proteins are expressed in a stable form, I can perform similar experiments as described for the bub1-1207 strain to further characterize the checkpoint defects in bub1-1221 and bub3-1208.

Summary of Work Described Previously Pertaining to Task 4 of Specific Aim 1:
I identified strain 4101 from the *mph1* overexpression screen as a mutant that is sensitive to thiabendazole, temperature sensitive, and survives overexpression of *mph1*. I performed three independent experiments to confirm that strain 4101 is deficient in the spindle checkpoint because it can not arrest the cell cycle in response to spindle disruption or microtubule damage. By microscopic analysis of strain 4101 after 4 hours at the restrictive temperature, I observed that 4101 mutant cells undergo an abnormal mitosis as evidenced by the following phenotypic observations: chromosome missegregation, lagging chromosomes, cell division offset from the center of the cell, and condensed DNA that is stretched across the center of the cell. These observations are consistent with spindle checkpoint deficiency because proceeding through the cell cycle before kinetochores are properly attached to the mitotic spindle could promote the observed phenotypes.

I previously crossed strain 4101 to strains carrying null mutations of the known spindle checkpoint genes in *S. pombe (mad1, mad2, mad3, bub1,* and *bub3)* (Bernard et al., 1998; He et al., 1998; He et al., 1997; Ikui et al., 2002; Millband and Hardwick, 2002), and determined that the temperature sensitive mutation in strain 4101 is not located in one of these known spindle

checkpoint genes, indicating that strain 4101 may be mutated in a novel spindle checkpoint gene.

Current Results Pertaining to Task 4 of Specific Aim 1:
Because the mutation in strain 4101 is recessive, plasmid rescue of the 4101 temperature sensitive phenotype can be utilized to identify the mutated gene (Statement of Work, Task 4). However, my previous efforts to identify the gene mutated in strain 4101 by this strategy were unsuccessful. Listed below are three reasons this strategy may have been unsuccessful and the alternatives I am currently employing to circumvent these problems.

(1) Although the 4101 strain is temperature sensitive when compared to a wild type strain, it is capable of some residual growth at the restrictive temperature. I tested a number of approaches to optimize the conditions for plasmid rescue. I observed that the 4101 mutant does not grow in the presence of 15μg/mL thiabendazole at the restrictive temperature. I have used these more stringent conditions to screen library transformants to identify plasmids which may contain the gene mutated in strain 4101. Four rescued transformants have been obtained using this strategy.

(2) The gene mutated in strain 4101 may not be represented in the pURSP1 genomic library that was used in previous transformations (Barbet et al., 1992). This genomic library was made by performing partial digests using the restriction enzyme, Sau3A, which has approximately 1 recognition site every 450 nucleotides in the *S. pombe* genome. The pURSP1 library contains inserts ranging from 2.5 to 10 Kb with an average insert size of 3.1 Kb. It is possible that this library does not contain the gene mutated in strain 4101 because I have not been able to identify transformed strains which are significantly more temperature resistant or TBZ-resistant than vector controls. Therefore, I have used a different genomic library, called SP5, to transform the 4101 strain and screen for strains which are resistant to temperature or TBZ. Like the pURSP1 library, the SP5 genomic library was constructed by Sau3A partial digest. However, the SP5 library may be more representative of the *S. pombe* genome because it contains inserts ranging from 3 to 12 Kb with an average insert size of 3.3 Kb (McKenzie et al., 1987). I screened 34,000 transformants from the SP5 library by growing all library transformants at the permissive temperature and replica plating to the restrictive temperature on plates containing phloxine B, a vital dye that accumulates in dying cells. Transformants were also replica plated to plates containing TBZ and phloxine B. These conditions allow the identification of colonies which are temperature resistant or TBZ-resistant because they do not accumulate the phloxine B dye. Six rescued transformants have been identified using this strategy.

(3) It is possible that a plasmid carrying the gene mutated in strain 4101 would be toxic to 4101 mutant cells at the permissive temperature but not at the restrictive temperature because overexpression toxicity would be less pronounced at the restrictive temperature when the 4101 mutant protein is destabilized. In this case, it may not be possible to obtain transformants which carry the 4101 gene if they are initially grown at the permissive temperature. To address this possibility, I incubated 4101 cells transformed with the SP5 library immediately at the restrictive temperature. I then replica plated to plates containing phloxine B, incubated at the restrictive temperature, and selected colonies which do not accumulate this vital dye. I screened 34,000 transformants and identified four rescued transformants.

Currently, I am isolating the plasmids from the rescued transformants and transforming them back into the 4101 strain to determine if the plasmids confer resistance to temperature or TBZ. In addition, I am examining the rescued transformants to determine if the isolated plasmids rescue the mitotic abnormalities observed in the 4101 mutant. Plasmids that rescue the 4101 mutant phenotypes will be integrated into the genome, and linkage analysis and Southern blotting will be used to determine if any of these plasmids contain the gene that is mutated in strain 4101 or a suppressor that may function in the spindle checkpoint pathway. If the gene mutated in strain 4101 is a novel *S. pombe* gene, I will delete this open reading frame and characterize the spindle checkpoint defects and mitotic defects in the deletion mutant. If the gene mutated in strain 4101 is a gene that has been characterized in *S. pombe* but has not been implicated in the spindle checkpoint, I will determine if the known role of this protein affects the spindle checkpoint or if the protein has a separate spindle checkpoint function. I will also identify which nucleotides are altered in the 4101 mutant gene and determine how this alteration affects the function of the 4101 protein.

Specific Aim 2: Identify the functional regions of the human Mad2 protein.

Mad2p binds directly to Mad1p and to Cdc20p (Chen et al., 1999; Fang et al., 1998; Hwang et al., 1998; Ikui et al., 2002; Kallio et al., 1998; Kim et al., 1998). The interaction between Mad2p and Mad1p is necessary for the recruitment of Mad2p to kinetochores that are not attached to the mitotic spindle and for activation of the spindle checkpoint (Chen et al., 1999; Chen et al., 1998). Mad2p interaction with Cdc20p is required for the metaphase arrest that results from spindle checkpoint activation because the Mad2p-Cdc20p complex inhibits the ubiquitin ligase activity of the Anaphase Promoting Complex (APC) (Fang et al., 1998; Wassmann and Benezra, 1998). Therefore, mutations in the *mad2* gene which alter Mad2p residues that are important for binding to Mad1p or Cdc20p would likely inactivate the spindle checkpoint. It may be possible to identify such inactivating mutations using *S. pombe*. Previous work in the Sazer lab has established that *hsMAD2* can rescue the thiabendazole-sensitive phenotype of *S. pombe mad2* null cells (Wassman, Ong, Benezra, and Sazer, unpublished results). In my original application, I proposed to mutagenize *hsMAD2* (Statement of Work, Task 1) and introduce it into *mad2* null cells in order to identify mutations that inactivate *hsMAD2* (Statement of Work, Tasks 2-4) and would be likely to cause inactivation of the spindle checkpoint and chromosomal instability in human cells.

Initiation of work on Specific Aim 2 was to begin in November of 2001 according to the approved Statement of Work. However, prior to that time, the functional region of the human Mad2 protein necessary for the Mad2p-Cdc20p interaction had been identified by nuclear magnetic resonance (NMR) spectroscopy (Luo et al., 2000). In this study, the authors calculated the solution structure of the Mad2p monomer and the structure of Mad2p complexed with $Cdc20_{111-150}$. The authors were able to identify the functional regions of Mad2p which are necessary for its interaction with Cdc20p. The authors also identified specific residues, mainly in the C-terminal tail of Mad2p, which are likely important for its structural rearrangement upon binding to Cdc20p.

It has also been shown that the other known Mad2p-binding protein, Mad1p, promotes changes in the tertiary structure of Mad2p which are similar to the structural changes which occur when Mad2p binds Cdc20p (Luo et al., 2002). Recently, the crystal structure of a tetrameric Mad1p-Mad2p core complex was solved (Sironi et al., 2002). This study confirmed that the C-terminal tail

of Mad2p undergoes the same dramatic rearrangement when Mad2p is bound to Mad1p or to Cdc20p. Specifically, residues 161-205 were involved in the conformational changes that accompany binding of ligand to Mad2p.

Mad1p and Cdc20p are the only known proteins which directly bind to Mad2p. Published studies have already identified functional regions and specific residues of the Mad2 protein which are essential for its interaction with Mad1p and Cdc20p, and therefore are essential for checkpoint function. In addition, these studies have identified residues which are likely important for the structural rearrangements of the Mad2p protein which occur upon binding to its ligands. Therefore, these studies have already identified the functional regions of the Mad2 protein, which was the goal of Specific Aim 2. It is possible that the experiments outlined in Specific Aim 2 could identify additional Mad2p residues which are important for spindle checkpoint function. However, this screen would also identify *hsMAD2* mutants which are checkpoint deficient because of mutations in the structural core of the protein that result in misfolding. Such mutants would not be helpful in defining functional domains of the Mad2 protein. Therefore, it is unlikely that the experiments described in Specific Aim 2 would contribute to our understanding of the functional domains of the Mad2 protein, and I am asking permission not to perform these experiments.

Because the goals of Specific Aim 2 have been achieved by others, I have been able to begin work on Specific Aim 3 two months ahead of schedule.

Specific Aim 3: Identify new components of the mammalian spindle checkpoint pathway.

Although the spindle checkpoint is not essential in yeast, this pathway is essential in higher eukaryotes and mammals. In mice, Mad2p and Bub3p are required for embryonic viability even in the absence of spindle damage (Dobles et al., 2000; Kalitsis et al., 2000). Early embryos lacking Mad2p or Bub3p appear to lose viability due to the accumulation of mitotic errors including chromosome missegregation events. These and other studies (Basu et al., 1999; Gorbsky et al., 1998; Kitagawa and Rose, 1999) have indicated that higher eukaryotes require the spindle checkpoint in every mitotic division to ensure that anaphase does not proceed until all chromatids are properly attached to a functional spindle. To date, all mammalian checkpoint proteins which function in the spindle checkpoint signaling pathway were identified as homologues of yeast checkpoint proteins. Since the spindle checkpoint is essential in higher eukaryotes, it is expected that higher eukaryotes and mammals have evolved a more sophisticated pathway to protect against mitotic errors. In support of this idea, the human genome contains at least two proteins with similarity to yeast Mad2p (Cahill et al., 1999). Overproduction of human Mad2p, like overproduction of *S. pombe* Mad2p, causes a metaphase cell cycle arrest in fission yeast (Wassmann, Ong, Benezra, and Sazer, unpublished results). Therefore, fission yeast genetic approaches can be used to identify mammalian spindle checkpoint genes. The successful overproduction toxicity screen which led to the identification of the *S. pombe* spindle checkpoint proteins, Mad2p and Mph1p, can be repeated using a mammalian cDNA library to find previously unknown components of the mammalian spindle checkpoint pathway. A mammalian cDNA library has been constructed which allows mouse cDNAs to be overexpressed from a regulatable promoter in fission yeast (Craig and Norbury, 1998). Colonies which are sensitive to overexpression of the cDNA can be identified by retention of the vital dye phloxine B in dead cells. Cells from these colonies can then be examined cytologically to determine if the toxicity due to overexpression is likely caused by a cell cycle arrest

at metaphase, the point where activation of the spindle checkpoint stops cell cycle progression. An identical strategy was used to identify the first two spindle checkpoint genes in *S. pombe*. Strains overexpressing *S. pombe mad2* or *mph1* lose viability and exhibit hypercondensed DNA and an increase in the percentage of septated cells with an undivided nucleus and a short mitotic spindle (He et al., 1998; He et al., 1997).

Before initiating this screen, it was first necessary to determine the best conditions for identifying transformants which exhibit characteristics of a metaphase arrest. In the original screen which identified *mad2* and *mph1*, colonies which accumulated phloxine B and were enriched in septated cells when the cDNA was overexpressed were examined cytologically to determine if they had an undivided nucleus. This is an indirect method of screening for cells that were temporarily arrested in metaphase but have now septated and exited mitosis without completing nuclear division. It was later determined that these cells were indeed arrested in metaphase by overexpressing the cDNA in growing cells and observing an accumulation of cells with a short metaphase spindle, as revealed by staining with an anti-tubulin antibody. Since submission of my original proposal, I have devised an improved method that will allow me to directly screen cDNA transformants for the presence of short mitotic spindles, which is indicative of cDNAs that promote a metaphase arrest and therefore may encode spindle checkpoint proteins. This will allow me to screen a much larger number of strains which are sensitive to overexpression of a mammalian cDNA because it does not require the cells to be fixed, digested, and stained with antibodies in order to visualize the microtubules. The screen will therefore be carried out in a fission yeast strain which carries an integrated copy of the Green Fluorescent Protein (GFP) fused to the α-tubulin gene (GFP-atb2) (Ding et al., 1998). In this strain, microtubules can be visualized in living cells, and cells that are arrested in metaphase are readily identifiable because they have a short, easily visualized mitotic spindle but do not have cytoplasmic microtubule bundles(See Figure 3). To test this approach, I transformed the *S. pombe* GFP-atb2 strain with a vector containing *S. pombe mad2* or *mph1* under the control of the thiamine-repressible *nmt1* promoter (Maundrell, 1993). As expected, transformants overexpressing *mad2* or *mph1* accumulated phloxine B because overexpression of either *mad2* or *mph1* activates the spindle checkpoint, causes cells to arrest in metaphase, and is toxic to cells (He et al., 1998; He et al., 1997). Cytologically, I observed that overexpression of *mad2* or *mph1* results in an increase in the number of cells which are elongated, septated, or contain a short mitotic spindle (see Figure 3). I determined the percentage of cells with a short mitotic spindle or septated cells with an undivided nucleus present in strains overexpressing *mad2* or *mph1* (see Figure 4). I observed that 23.6% of cells overexpressing *mph1* and 21.3% of cells overexpressing *mad2* have a short mitotic spindle 16 hours after inducing expression of *mph1* or *mad2*. Under the same conditions, only 2.8% of vector control cells have a short mitotic spindle. I observed that 12.0% of cells overexpressing *mph1* and 2.0% of cells overexpressing *mad2* are septated with an undivided nucleus 18 hours after induction of *mph1* or *mad2*. This phenotypic class of cells was never observed in the vector control sample. In addition, I observed a decrease in the percentage of binucleate cells in strains overexpressing *mph1* or *mad2*, an indication that these cells are not going through mitosis (see Figure 4). 4.3% of cells overexpressing *mph1* and 2.7% of cells overexpressing *mad2* are binucleate while 16.0% of vector control cells are binucleate 18 hours after inducing expression of *mph1* or *mad2*.

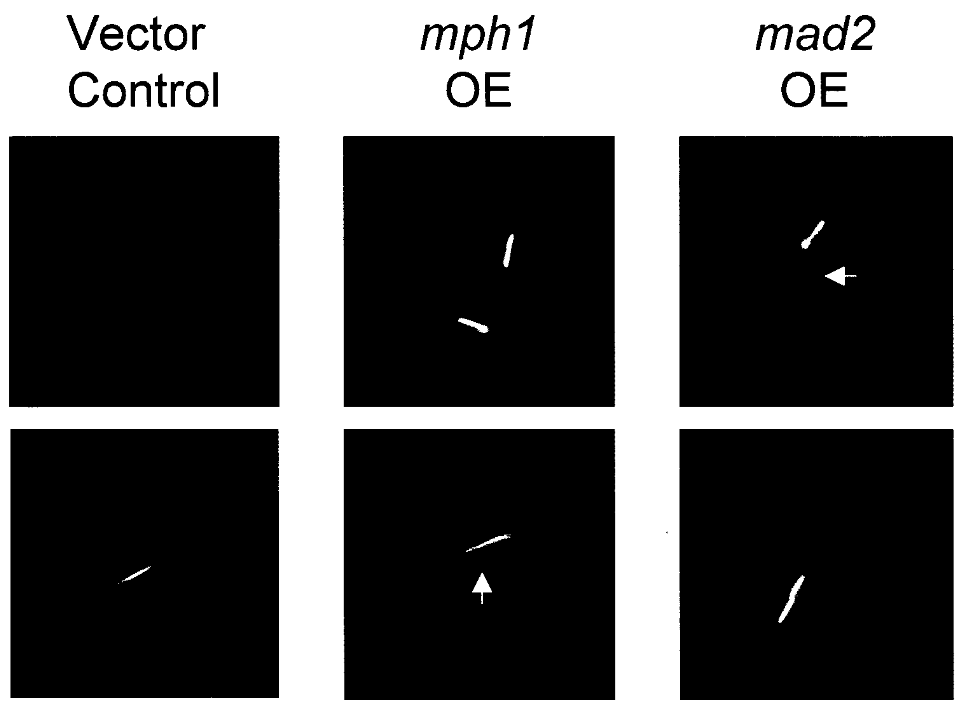


Figure 3: Wild type *S. pombe* cells overexpressing *mph1* or *mad2* are enriched in septated, elongated cells with short mitotic spindles. An *S. pombe* GFP-α-tubulin strain (GFP-atb2) was transformed with the indicated vectors in the presence of thiamine to repress expression of *mph1* or *mad2*. After 5 days incubation at 32˚C, overexpression (OE) of *mph1* or *mad2* was induced by replica plating to plates that do not contain thiamine. Colonies were examined cytologically after a 24 hour incubation at 32˚C in the absence of thiamine. The position of the septum is indicated by an arrow.

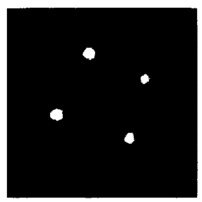
		Vector Control	*mph1* OE	*mad2* OE
Short mitotic spindles		2.8%	23.6%	21.3%
Septated cells, undivided nucleus		0.0%	12.0%	2.0%
Binucleate cells		16.0%	4.3%	2.7%

Figure 4: Wild type cells overexpressing *S. pombe mph1* or *mad2* are enriched in cells with short mitotic spindles and septated cells with an undivided nucleus and have fewer binucleate cells. Cells transformed with the indicated plasmids were grown for 24 hours at 32°C in the presence of thiamine. Overexpression (OE) of the indicated genes was induced by washing the cells and growing them in the absence of thiamine at 32°C. Cells were scored for the presence of a short mitotic spindle by examining live cells after 16 hours incubation in the absence of thiamine. Septated cells with an undivided nucleus and binucleate cells were scored by examining ethanol fixed cells stained with DAPI to visualize DNA after 18 hours incubation in the absence of thiamine. At least 300 cells were scored for each sample.

I will now perform a screen to identify mammalian cDNAs which induce a metaphase arrest in *S. pombe* (see Figure 5). *S. pombe* GFP-atb2 cells will be transformed with a mouse cDNA library (Statement of Work, Task 1). The mouse cDNA and the GFP-*atb2* gene are both under the control of the same thiamine-repressible *nmt1* promoter. Transformants will be grown on plates containing thiamine to repress expression of the cDNA. Transformants which exhibit decreased viability when the cDNA is overexpressed will be identified by accumulation of phloxine B in the absence of thiamine (Statement of Work, Task 2). The absence of thiamine in these plates also induces expression of GFP-*atb2*, so colonies which exhibit poor growth when the cDNA is overexpressed can be examined cytologically to determine if they are enriched in elongated cells with short mitotic spindles (Statement of Work, Task 3). Plasmids from selected strains which arrest in metaphase when the cDNA is overexpressed will be isolated, retransformed to confirm their overexpression phenotype, and sequenced to identify the potential mammalian spindle checkpoint genes (Statement of Work, Task 5). Although beyond the scope of this proposal, the requirement of these potential mammalian spindle checkpoint genes can be tested in mouse and human cell lines. It can then be determined if these genes are mutated in human tumors and tumor cell lines. In addition, since the mouse cDNA causes a metaphase arrest in *S. pombe*, studies can be performed in fission yeast to determine how the new checkpoint proteins interact with known components and activate the spindle checkpoint to induce the cell cycle arrest.

Transform an *S. pombe* GFP-α-tubulin expressing strain with a mouse cDNA library.

↓

Select for library transformants in the presence of thiamine to repress expression of the cDNA.

↓

Replica plate to plates that do not contain thiamine to induce expression of the mouse cDNA and GFP-α-tubulin.

↓

Identify colonies that lose viability when the mouse cDNA is overexpressed.
Examine cells from these colonies microscopically.

↓

Select colonies which are enriched in elongated cells with short mitotic spindles.

↓

Examine cells grown in liquid to confirm that the cDNA causes cells to arrest in metaphase.

↓

Isolate and sequence mouse cDNAs which induce a metaphase arrest in *S. pombe*.

Figure 5: Strategy of the screen to identify mouse cDNAs that induce a metaphase arrest when overexpressed in *S. pombe*. *S. pombe* cells expressing GFP-α-tubulin will be transformed with a mouse cDNA library. Transformants will be grown on plates containing thiamine to repress expression of the cDNA. Transformants which exhibit decreased viability when the cDNA is overexpressed will be identified as colonies that accumulate phloxine B in the absence of thiamine. Colonies enriched in elongated cells with short mitotic spindles will be chosen for further study. Plasmids from these selected strains will be isolated and sequenced to identify the potential mammalian spindle checkpoint genes.

KEY RESEARCH ACCOMPLISHMENTS

- Four strains that do not arrest the cell cycle in response to *mph1* overexpression are mutated in a known spindle checkpoint gene. The *bub1* allele in bub1-1207 is predicted to encode a Bub1p that is truncated in the highly conserved protein kinase domain (see Figure 1). The *bub1* allele in bub1-1221 is predicted to encode a protein that is mutated in the conserved Mad3-like region of Bub1p (see Figure 1). The *bub3* allele in bub3-1208 is predicted to encode a Bub3p that is mutated in the most carboxy terminal WD repeat domain. I am currently amplifying and sequencing the *mad1* allele in mad1-1201.

- Of the known spindle checkpoint genes described above, only bub1-1207 exhibits a phenotype that is distinct from its deletion mutant. The bub1-1207 strain is approximately 25 times more TBZ-sensitive than the bub1Δ strain (see Figure 2). Further studies will characterize the checkpoint defect in this strain.

- Previous efforts to identify the gene mutated in the potentially novel spindle checkpoint mutant, strain 4101, were unsuccessful. Potential problems with the original strategy have been addressed, and 14 plasmids were identified as rescuers of the temperature sensitivity or TBZ-sensitivity of strain 4101. Current efforts focus on determining if any of these plasmids contain the gene mutated in strain 4101 or if they contain a suppressor of the 4101 mutation that functions in the spindle checkpoint pathway.

- The goals of Specific Aim 2, to identify the functional regions of the Mad2 protein, have been achieved by others. Therefore, I am asking permission not to perform these experiments, and I have initiated work on Specific Aim 3 two months ahead of schedule.

- Overexpression of *S. pombe mad2* or *mph1* in cells expressing GFP–α-tubulin results in an enrichment of elongated cells with a short mitotic spindle, which is indicative of a cell cycle arrest at metaphase (See Figures 3 and 4).

- The GFP-α-tubulin strain will be used to perform the screen outlined in Specific Aim 3 (See Figure 5). The use of this strain is an improvement to my original proposal which will enable me to screen more mammalian cDNAs to determine if they induce a metaphase arrest in *S. pombe*.

REPORTABLE OUTCOMES

(1) The following abstract was submitted to the 2000 FASEB Yeast Chromosome Structure Meeting held in Snowmass Village, Colorado. My abstract was accepted, and I presented a poster at this meeting.

The spindle assembly checkpoint in fission yeast

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During anaphase of mitosis, sister chromatids are separated by the dynamic microtubules which comprise the mitotic spindle. Failure to achieve equal distribution of the chromosomal DNA can lead to genetic loss and lethality. In eukaryotic cells, the spindle assembly checkpoint monitors the structural integrity of the spindle and initiates a cell cycle delay to allow for spindle repair. The fission yeast, *Schizosaccharomyces pombe*, is a useful system for discovering and characterizing components of this regulatory pathway because genetic approaches can be coupled with excellent cytology. In *S. pombe*, spindle checkpoint mutants can be identified by their sensitivity to the microtubule destabilizing drug, thiabendazole (TBZ). Wild type cells arrest the cell cycle in response to the drug and repair spindle damage before anaphase while checkpoint mutants fail to arrest and undergo an aberrant division that results in cell death. Previous work in our lab identified the first two spindle checkpoint genes in *S. pombe*: *mad2* and *mph1*. Overexpression of either *mad2* or *mph1* activates the spindle checkpoint, arrests cells prior to anaphase, and is toxic to wild type cells. *mph1* acts upstream of *mad2*, and the toxic effect of *mph1* overexpression is suppressed by a *mad2* deletion.

This project aims to discover and characterize novel components of the spindle checkpoint pathway by identifying other suppressors of *mph1* overexpression. Several lines of evidence indicate that this screen has produced spindle checkpoint mutants: the strains are sensitive to TBZ; three of twenty strains tested to date carry mutations in known spindle checkpoint components; and temperature sensitive strains display lagging chromosomes and missegregation of DNA at the restrictive temperature. The identification of the gene mutated in selected strains will increase our understanding of the spindle checkpoint by allowing characterization of new components of this pathway.

(2) A manuscript entitled "A genetic screen to identify spindle checkpoint deficient strains in *Schizosaccharomyces pombe*" is in preparation.

CONCLUSIONS

The *mph1* overexpression screen has identified four strains mutated in known spindle checkpoint genes. The bub1-1207 strain contains a mutant *bub1* allele that is predicted to encode a Bub1p that is truncated in the highly conserved protein kinase domain. The bub1-1221 strain contains a mutation in *bub1* which is predicted to encode a protein that is mutated in the conserved Mad3-like region of Bub1p. The mutant *bub3* allele in bub3-1208 is predicted to encode a Bub3p that is mutated in the most carboxy terminal WD repeat domain. Currently, I amplifying and sequencing the *mad1* allele in mad1-1201 to identify the mutation.

The bub1-1207 strain has a phenotype that is distinct from a bub1Δ strain as indicated by the decreased ability of the bub1-1207 strain to grow in the presence of thiabendazole (TBZ) when compared to the bub1Δ strain. The *bub1* gene in bub1-1207 is mutated in the highly conserved Bub1p kinase domain. Further studies will characterize the spindle checkpoint defect in bub1-1207 and address the effect of the mutation on the ability of the Bub1p to interact with other spindle checkpoint components, bind to kinetochores, and affect the recruitment of spindle checkpoint proteins to kinetochores when the checkpoint is activated. No published studies have reported the identification of a fission yeast *bub1* allele that is phenotypically distinct from a bub1Δ strain. Therefore, the bub1-1207 strain will be useful in further investigating the role of Bub1p in the *S. pombe* spindle checkpoint pathway.

The *mph1* overexpression screen has also identified a strain, called 4101, which is mutated in a potentially novel component of the spindle checkpoint pathway. Because the mutation in strain 4101 was shown to be recessive, plasmid rescue of the temperature sensitivity and TBZ-sensitivity is being utilized to identify the gene mutated in strain 4101. However, previous efforts to identify the gene mutated in strain 4101 using this strategy have been unsuccessful because strong rescuers of the temperature sensitivity or TBZ-sensitivity have not been identified. It is possible that the gene mutated in strain 4101 is not represented in the genomic library, that residual growth of the 4101 strain at the restrictive temperature has made it difficult to identify rescued strains, or that the gene mutated in strain 4101 is toxic when expressed from a multicopy plasmid at the permissive temperature. Several new strategies were devised and employed to address these problems, and 14 plasmids which rescue the temperature sensitivity or TBZ-sensitivity of strain 4101 have been identified. After determining which of these plasmids best rescue all of the 4101 mutant phenotypes, it will be determined if these plasmids encode the gene mutated in strain 4101 or a high copy suppressor which may function in the spindle checkpoint pathway.

Although the spindle checkpoint is not essential in yeast, this pathway is essential in higher eukaryotes and mammals (Dobles et al., 2000; Gorbsky et al., 1998; Kalitsis et al., 2000; Basu et al., 1999; Kitagawa and Rose, 1999). To date, all mammalian checkpoint proteins which function in the spindle checkpoint signaling pathway were identified as homologues of yeast checkpoint proteins. However, since the spindle checkpoint is essential in mammals, it is expected that mammals have evolved a more sophisticated pathway to protect against mitotic errors and therefore may have spindle checkpoint proteins that are not present in yeast. Overproduction of human Mad2p, like overproduction of *S. pombe* Mad2p, causes a metaphase cell cycle arrest in fission yeast (Wassmann, Ong, Benezra, and Sazer, unpublished results). Therefore, fission yeast genetic approaches can be used to identify mammalian spindle

checkpoint genes by identification of cDNAs that are toxic when overexpressed in *S. pombe* and cause cells to arrest in metaphase. The original strategy submitted for Specific Aim 3 has been revised to allow easier identification of strains that are arrested in metaphase by performing the cDNA library transformation in an *S. pombe* strain expressing GFP-α-tubulin. In this strain, cells overexpressing *S. pombe mad2* or *mph1* accumulate elongated cells with short mitotic spindles. Plasmids which promote a metaphase arrest in *S. pombe* will be isolated, retransformed into *S. pombe* to confirm the overexpression phenotype, and sequenced to identify the potential mammalian spindle checkpoint genes. Once potential mammalian spindle checkpoint genes have been identified, the requirement of these genes for spindle checkpoint function can be tested in mouse and human cell lines, and analysis of human tumors and human tumor cell lines can be performed to determine if the new checkpoint genes are mutated in human cancers. In addition, further studies in *S. pombe* can be performed to determine how the new mammalian checkpoint proteins activate the spindle checkpoint pathway to promote a cell cycle arrest in fission yeast.

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